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In-vitro toxoplasmacidal activity of cationic electron carriers

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Exposing murine macrophages infected with the protozoan parasite *Toxoplasma gondii* to micromolar concentrations of some cationic electron carriers (dyes), resulted in complete killing of the intracellular parasites at concentrations at which these compounds did not seem toxic for the macrophages. The 50% inhibitory concentrations (with 95% confidence limits) were calculated as 0·26 (0·18–0·37), 1·35 (1–2·25), 0·45 (0·13–1·50), and 1·52 (0·91–2·53) μM for crystal violet, phenazine methosulphate, methylene blue and brilliant cresyl blue, respectively. The effects of these electron carriers did not appear to be the result of an enhancement of the natural antitoxoplasmic activity of the macrophages. None of the tested compounds was active against extracellular *Tox. gondii* as measured by ability to reinfect murine macrophages; thus, these dyes seem to act primarily on actively metabolizing, intracellular, *Tox. gondii*. Our data also suggest that the killing effect of the electron carriers was not related to the generation of reactive oxygen intermediates as judged by the inability of scavengers of these intermediates to block the activity against intracellular *Tox. gondii*. Further studies with related redox compounds would have an interesting chemotherapeutic perspective for treating toxoplasma infections.

Introduction

Toxoplasma gondii is a ubiquitous protozoan parasite which can cause disease in man. No satisfactory cidal therapy is available for treating life-threatening toxoplasma infections which can occur among patients with the Acquired Immune Deficiency Syndrome (AIDS).

It seems to be well established that components of the cellular immune response (mononuclear phagocytic system) are the main effectors of defence against this obligate intracellular parasite. Activated macrophages are able to kill *Tox. gondii in vitro*, and this killing seems to be associated with the ability of the macrophages to release reactive oxygen intermediates (ROI) such as superoxide anion, hydrogen peroxide and hydroxyl radical (Nathan *et al.*, 1983; Murray, Spitalny & Nathan, 1985). Studies have shown that cationic electron carriers, which are thought to undergo redox-cycling and generating superoxide anion and hydrogen peroxide (Hassan & Fridovich, 1979), were able to inhibit intracellular *Trypanosoma cruzi* and *Leishmania* spp. (Rabinovitch *et al.*, 1982; Alves & Rabinovitch, 1983; Mauel, Schnyder & Baggiolini, 1984). One of these electron carriers, the triarylmethane dye crystal violet (gentian violet), was shown to be active against extracellular *Tryp. cruzi in vitro* (Nussenzweig *et al.*, 1953). Since then, this dye has been used by blood banks in Latin-American endemic countries in an attempt to reduce the transmission of Chagas' disease. The mechanism of action of

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these compounds, however, has not been elucidated. Here, the in-vitro activity of cationic electron carriers against extracellular and intracellular tachyzoites of *Tox. gondii* was investigated.

Materials and methods

Animals

Female Swiss-Webster mice (Madörin, Füllinsdorf, Switzerland), weighing 23 to 25 g each, were used in all experiments.

Macrophages

Animals were killed by CO_2 asphyxiation, their resident peritoneal macrophages were harvested from their peritoneal cavities as described previously (Chang & Pechère, 1988), and the concentration of mononuclear cells was adjusted to 2×10^6/ml with medium 199 (M199) supplemented with 10% fetal calf serum (FCS) (pH 7·2). Each well of a 96-well tray (Costar, Cambridge, Massachussetts), or each chamber of four-chamber Lab-Tek slides (Lab-Tek Div., Miles Laboratories Inc., Naperville, Illinois), was seeded with 2×10^5 and 2×10^6 cells, respectively. They were incubated for 2–3 h at 37°C in a 5% CO_2-95% air humid atmosphere. The nonadherent cells were discarded by washing twice with prewarmed Hanks' balanced salt solution (HBSS).

Electron carriers

The following electron carriers were obtained from Fluka AG, Buchs, Switzerland: brilliant cresyl blue (mol. wt, 385·96), crystal violet (mol. wt, 407·99), methylene blue (mol. wt, 319·86) and phenazine methosulfate (mol. wt, 306·34). They were dissolved in culture medium and sterilized by filtration immediately before use.

Scavengers of oxygen intermediates

Scavengers of oxygen intermediates (Catterall, Sharma & Remington, 1986) were obtained from Sigma GmbH, Deisenhofen, West Germany, and used at the concentrations shown. Superoxide dismutase, 3000 U/mg (2·5 mg/ml) was used for superoxide anion; catalase from bovine liver recrystallized twice (2·5 mg/ml) for hydrogen peroxide; and histidine (10 mM) and diazabicyclooctane (1 mM) for singlet oxygen radicals. The scavengers of hydroxyl radicals were benzoic acid (10 mM), mannitol (50 mM) and tetramethylurea (25 mM). These scavengers were added to the monolayers immediately after the 1 h challenge with *Tox. gondii* and 30 min before the addition of the cationic electron carriers. All these experiments were performed over a 24-h period of incubation.

Tox. gondii *infection and microbicidal assays*

Cell cultures were challenged for 1 h with 2×10^5 (96-well trays) or 2×10^6 (Lab-Tek slides) RH strain *Tox. gondii* tachyzoites. After uningested parasites had been removed by washing, standard medium containing reagents was added and the monolayers were

pulsed with 2·5 μCi of [5-6-^{3}H]uracil (specific activity 49 Ci/mmol; Amersham plc, Buckinghamshire, England). After a 24-h period of incubation the radioactivity was counted in the acid-precipitable material by filtration procedure (Chang & Pechère, 1988). The inhibition of the intracellular *Tox. gondii* growth rate was thus assessed by using [^{3}H]uracil, a precursor which is incorporated by *Tox. gondii*, but not the host cells (Chang & Pechère, 1988). In addition, the outcome of intracellular infection was assessed by microscopically counting the number of infected cells and the number of intracellular parasites per 100 cells in Giemsa-stained preparations. An antitoxoplasmic effect was indicated by a decrease in the parameters considered at 24 h.

Effects of electron carriers on extracellular toxoplasmas

Fresh suspensions of *Tox. gondii* (3–6 $\times 10^6$ tachyzoites/ml) were incubated in 5% CO_2-95% air at 37°C with the electron carriers for 30 min at the desired concentrations. The suspensions were centrifuged at 50 *g* for 10 min, washed with phosphate-buffered saline (pH 7·2), centrifuged again and resuspended in M199 with 3% FCS. They were used to challenge macrophage monolayers for [^{3}H]uracil experiments and for Giemsa-stained preparations.

Effect of electron carriers on antitoxoplasmic activity of resident macrophages

Mouse macrophage monolayers were exposed for 24 h to the electron carriers at the desired concentrations. At the end of the incubation period, the monolayers were washed three times with prewarmed HBSS and challenged with freshly harvested *Tox. gondii* for 1 h. Assessment of *Tox. gondii* inhibition was made after a 24-h incubation period in medium without the electron carriers.

Assessment of toxicity on macrophages

Adherent macrophage monolayer cells were incubated for 24 h with different concentrations of the electron carriers and their viability was assessed as described previously (Chang & Pechère, 1988), by the trypan blue dye exclusion test. In addition, macrophage monolayers were examined in Giemsa-stained preparations, after 24 h incubation, for the number of cells per high power field and assessed for their morphological characteristics.

Statistics

Data are reported as the mean $\pm$ standard error of the mean (SEM). The 50% inhibitory concentrations (IC_{50}), the 95% confidence limits, and the IC_{90} were calculated by probit analysis following the method of Litchfield & Wilcoxon (1949). differences between values were analyzed by the Student's *t*-test. A P value $<0·05$ was considered significant.

Results

Activity of electron carriers on intracellular toxoplasmas

Infected murine peritoneal macrophages were exposed to increasing amounts of the electron carriers in concentrations from 0·001 to 35 μM, and the growth rate of

Table I. Activity of cationic electron carriers against intracellular *Tox. gondii* as assessed by [^{3}H]uracil assay

Treatment of infected macrophages	50% Inhibitory concentration (IC_{50}) (μM) (95% fiducial range)	90% Inhibitory concentration (IC_{90}) (μM)
Crystal violet	0·26 (0·18–0·37)	0·43
Phenazine methosulphate	1·35 (1·00–2·25)	2·38
Methylene blue	0·45 (0·13–1·50)	2·38
Brilliant cresyl blue	1·52 (0·91–2·53)	3·24

intracellular *Tox. gondii* was assessed by measuring the uptake of [^{3}H]uracil over a period of 24 h. Micromolar concentrations of all electron carriers tested were able to restrict the intracellular multiplication of the parasite. This allowed calculation of their IC_{50} and IC_{90} as shown in Table I. Table II summarizes the results of experiments performed with microscopically assessed preparations. All electron carriers at their respective IC_{90}, according to the [^{3}H]uracil incorporation studies, significantly reduced the number of infected cells and the number of toxoplasmas per 100 cells, and at higher concentrations allowed complete eradication of the parasites from the macrophages. These effects were seen at concentrations which were not toxic for the macrophages according to the trypan blue dye exclusion test and morphological criteria. By raising the concentrations, toxic effects on the cells however, appeared with increased concentrations of crystal violet, phenazine methosulphate and methylene blue (Table II).

Effect of electron carriers on extracellular toxoplasmas

The electron carriers did not alter the viability of extracellular *Tox. gondii*, assessed by their ability to reinfect macrophage cultures (Table III).

Table II. Activity of cationic electron carriers against intracellular *Tox. gondii* as assessed by microscopy

Treatment of infected macrophages with electron carriers (μM)		% of infected cells	Number of toxoplasmas per 100 cells	Minimal concentration for toxicity to macrophage monolayers (μM)[a]
Medium only	(control)	56±4	717±8	
Crystal violet	(0·43)	0[d]	0[e]	0·55
Phenazine methosulphate	(2·38)	8±1[b]	16±1[c]	7
	(5)	0[d]	0[e]	
Methylene blue	(2·38)	7±1[b]	11±1[c]	30
	(15)	0[d]	0[e]	
Brilliant cresyl blue	(3.24)	5±1[b]	11±1[c]	No toxicity up to 35
	(31)	0[d]	0[e]	

Results are the mean ±SEM of three experiments.

[a]Assessed by trypan blue dye exclusion and morphological criteria.

[b]$P < 0·01$, compared to control; [c]$P < 0·001$, compared to control; [d]$P < 0·0001$, compared to control; [e]$P < 0·00001$, compared to control.

Table III. Effect of cationic electron carriers on extracellular *Tox. gondii* as assessed by subculture

Pretreatment with electron carriers (μM)		% of infected cells	Number of toxoplasmas per 100 macrophages	Incorporation of [^{3}H]uracil[a]
Extracellular *Tox. gondii*				
None		49±1	629±6	100
Crystal violet	(0·43)	48±2	611±7	94±8·3
Phenazine methosulphate	(2·38)	53±2	665±9	98±9·1
Methylene blue	(2·38)	51±1	640±5	99±6·8
Brilliant cresyl blue	(3.24)	48±1	614±8	96±7·5

Results are the mean ±SEM of three experiments.
[a]Expressed as percentage of intracellular inhibition: (100-cpm in test/cpm in control) × 100.

Effect on anti-toxoplasma activity of resident macrophages

In order to test whether or not these compounds produced some degree of macrophage activation, we incubated resident macrophage monolayers with the different electron carriers for 24 h, and challenged them with *Tox. gondii*. None of the tested electron carriers was able to enhance the antitoxoplasmic activity of the resident mouse macrophages (Table IV).

Effect of scavengers of oxygen intermediates

We further investigated the effect of scavengers of oxygen intermediates on the activity of the cationic electron carriers at their corresponding IC_{90}. The scavengers of ROI did not block the ability of electron carriers to kill intracellular *Tox. gondii* at the concentrations tested (Table V).

Discussion

These studies showed that electron carriers were toxoplasmacidal *in vitro* at micromolar concentrations; the parasites were completely eradicated from the macrophages at some of the concentrations tested. The combination of pyrimethamine

Table IV. Effect of cationic electron carriers on antitoxoplasmic activity of macrophages

Pretreatment with electron carriers (μM)		% of infected cells	Number of toxoplasmas per 100 macrophages	Incorporation of [^{3}H]uracil[a]
Resident mouse macrophages				
None		47±1	597±8	100
Crystal violet	(0·43)	45±2	591±7	95±3·8
Phenazine methosulphate	(2·38)	51±2	628±9	93±7·6
Methylene blue	(2·38)	47±3	594±6	94±7·8
Brilliant cresyl blue	(3·24)	50±2	583±12	97±5·3

Results are the mean ±SEM of three experiments.
[a]Expressed as percentage of intracellular inhibition: (100-cpm in test/cpm in control) × 100.

Table V. Effects of scavengers of oxygen intermediates on the activity of cationic electron carriers against intracellular *Tox. gondii*[a]

Treatment of infected macrophages[b]	None	Crystal violet	Phenazine methosulphate	Methylene blue	Brilliant cresyl blue
Medium only	100	8·5±1·3	16·8±2·3	15·5±3·8	16·9±3·5
Catalase (2·5 mg/ml)	102±5·7	4·9±0·7	21·3±2·7	23·5±1·0	19·4±1·0
Superoxide dismutase (2·5 mg/ml)	96±6·9	4·9±0·8	24·8±3·0	17·3±3·2	25·5±3·1
Histidine (10 mM)	97±7·9	10·0±1·3	21·9±2·4	16·8±2·4	14·0±2·2
Diazabicyclooctane (mM)	94±9·7	6·8±1·9	18·1±2·6	19·7±3·5	13·7±0·5
Mannitol (50 mM)	99±5·2	10·7±2·1	13·0±3·5	19·7±3·6	17·5±0·8
Benzoic acid (25 mM)	97±6·8	5·7±0·9	11·2±1·6	18·3±2·1	17·8±1·0
Tetramethylurea (25 mM)	98±5·3	5·2±1·2	13·0±1·2	19·4±1·8	13·9±0·3

[a]Measured by [^{3}H]uracil assay, and expressed as percentage of intracellular inhibition as stated in Table III and IV.
[b]The electron carriers were added at their respective 90% inhibitory concentrations as shown in Table I.

and sulphadiazine, investigated in a similar system, was also active against *Tox. gondii* at the micromolar level, but exerted less killing effect (Chang & Pechère, 1988).

The electron carriers could have stimulated the macrophages to kill the parasites or could have acted directly on the intracellular parasites. Regarding a macrophage-mediated mechanism, some studies have suggested that the intracellular killing of leishmania by electron carriers, excepting crystal violet, correlated with the stimulation of the hexose monophosphate shunt, and with the generation of ROI (Mauel *et al.*, 1984). Here, stimulation of the release of ROI was unlikely since the antitoxoplasma activity of the electron carriers was not altered by the scavengers of ROI. Pretreatment of macrophages with electron carriers did not enhance the antitoxoplasmic activity of these cells, an observation which also tips the balance in favour of a direct antiparasitic effect. Since the electron carriers were not active against extracellular *Tox. gondii*, but only against actively growing intracellular parasites, an interference with the parasites' metabolism by the electron carriers is suggested. Some arguments for direct damage to the intracellular parasites can be found elsewhere. Phenazine methosulphate has been found to accumulate in the parasitophorous vacuoles of macrophages infected with leishmania (Rabinovitch *et al.*, 1982). A mechanism for a direct anti-*Tryp. cruzi* effect has been proposed for crystal violet, consisting in a photodynamic action apparently mediated by the formation of carbon-centred free radicals in the parasites themselves (Docampo *et al.*, 1983). An alternative mechanism could be direct oxidative damage to the parasites. In this view, a correlation of between toxoplasmacidal action and the redox potential of the electron carriers would have been expected. This was not the case, here, nor with other intracellular parasites also susceptible to the electron carriers (Rabinovitch *et al.*, 1982; Alves & Rabinovitch, 1983; Mauel *et al.*, 1984). However, such correlation is likely to be obscured by other structural requirements for drug permeation (Rabinovitch *et al.*, 1983; Mauel *et al.*, 1984).

In conclusion, we have shown that these electron carriers are toxoplasmacidal, but the mechanisms of their impressive activity remain unclear. Further investigation is warranted with these and related compounds because of their interesting chemotherapeutic perspective.

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